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(57) Abstract

The invention relates to a method of immortalizing a primary mammalian cell comprising overexpressing an Id protein in said cell, a method of inhibiting the immortalization of a primary mammalian cell comprising inhibiting expression of an Id protein in said cell and a method of determining the activity of a compound to alter Id protein expression in a cell comprising contacting said cell with said compound and observing the level of expression of said Id protein.

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IMMORTALIZATION OF PRIMARY HUMAN KERATINOCYTES BY THE HELIX-LOOP-HELIX PROTEIN, Id-1

BACKGROUND OF THE INVENTION

Basic helix-loop-helix (bHLH) DNA binding proteins have been demonstrated to regulate tissue-specific transcription within multiple cell lineages. The Id family of helix-loop-helix proteins does not possess a basic DNA binding domain and functions as a negative regulator of bHLH proteins.

SUMMARY OF THE INVENTION

The invention is based upon the discovery that Id-1, Id-2, and Id-3 can extend the normal lifespan of primary human keratinocytes. Furthermore, we demonstrate that Id-1 alone can immortalize primary human keratinocytes when overexpressed in these cells. Thus, immortalization of primary human cells can be achieved by overexpression of a single cellular oncogene. Based upon these results, bHLH proteins can play a pivotal role in regulating normal keratinocyte growth and differentiation which can be disrupted by the oncogenic functions of Id-1.

The invention relates to a method of immortalizing a primary mammalian cell comprising overexpressing an Id protein in said cell.

In a second embodiment the invention relates to a method of inhibiting the immortalization of a primary mammalian cell comprising inhibiting expression of an Id protein in said cell.

In another embodiment the invention relates to a method of determining the activity of a compound to alter Id protein expression in a cell comprising contacting said cell with said compound and observing the level of expression of said Id protein.

The foregoing and other objects, features and advantages of the invention will be apparent from the following more particular description of preferred

embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The differentiation program of multiple cell types has been shown to be dependent on the activity of basic helix-loop-helix (bHLH) transcription factors (reviewed in (Olson, E.N. and Klein, W.H., *Genes Dev. 8*:1-8 (1994); Weintraub, H., *Cell 75*:1241-1244 (1993)). These proteins share a common sequence motif of a stretch of basic amino acids responsible for site-specific DNA binding adjacent to a helix-loop-helix dimerization domain. The Id family of helix-loop-helix proteins, which does not possess a basic DNA binding domain, functions as a negative regulator of basic HLH proteins through the formation of inactive heterodimers with intact bHLH transcription factors (Benezra, R. *et al.*, *Cell 61*:49-59 (1990); Sun, X.H. *et al.*, *Mol. Cell Biol. 11*:5603-5611 (1991)).

A family of Id proteins has been demonstrated to bind the ubiquitously expressed E-proteins or cell lineage-restricted bHLH transcription factors leading to inhibition of lineage-specific gene expression and differentiation (Benezra, R. *et al.*, *Cell 61*:49-59 (1990); Wilson, R.B. *et al.*, *Mol. Cell Biol. 11*:6185-6191 (1991); Jen, Y. *et al.*, *Genes Dev. 6*:1466-1479 (1992); Kreider, B. *et al.*, *Science* :1700-1702 (1992)). Over the past few years, growing evidence has accumulated implicating Id proteins as playing a critical role in promoting G1-S cell cycle transitions. Id gene expression has been demonstrated to be elevated in undifferentiated cells and tumor cells supporting the notion of their role as inhibitors of differentiation and growth promoting factors (Ellmeier, W. *et al.*, *EMBO J. 11*:2563-2571 (1992); Zhu, W. *et al.*, *Res. 30*:312-326 (1995)). Overexpression of Id proteins within myoblasts, myeloid precursor cells, mammary epithelium, and preadipose cells has been shown to inhibit their ability to differentiate under appropriate conditions (Kreider, B. *et al.*, *Science* :1700-1702 (1992); Moldes, M. *et al.*, *Mol. Cell Biol. 17*:1796-1804 (1997); Jen, Y. *et al.*, *Genes Dev. 6*:1466-1479 (1992); Desprez, P.Y. *et al.*, *Mol. Cell. Biol. 1555*:3398-3404 (1995)). Id-2 has been determined to reverse cellular growth

inhibition by the retinoblastoma protein through direct interaction with pRb, p107, and p130 through its HLH domain (Iavarone, A. *et al.*, *Genes Dev. 8*:1270-1284 (1994); Lasorella, A. *et al.*, *Mol. Cell Biol. 16*:2570-2578 (1996)) and to abrogate a p16-mediated cell cycle arrest in Saos-2 cells (Lasorella, A. *et al.*, *Mol. Cell Biol. 16*:2570-2578 (1996)). Additionally, overexpression of E47 was demonstrated to induce a G1 cell cycle arrest in NIH 3T3 cells which was counteracted by overexpression of Id-1 (Peverali, F.A. *et al.*, *EMBO J. 13*:4291-4301 (1994)). More recently, E2A has been demonstrated to induce the expression of the cell cycle inhibitory protein, p21 (Prabhu, S. *et al.*, *Mol. Cell Biol. 17*:5888-5896 (1997)). This activity is suppressed by overexpression of Id-1.

Expression patterns of Id proteins during human keratinocyte differentiation have been investigated and it has been determined that they play a role in this process. Utilizing an *in vitro* human foreskin keratinocyte culture system previously described (Jones, D.L *et al.*, *Genes Dev. 11*:2101-2111 (1997); Pittelkow, M.R. *et al.*, *J. Investigative Dermatology 86*:410-417 (1986)). RNA was harvested from keratinocytes at various time points during differentiation. Nested PCR of total cellular RNA and Northern blotting demonstrated that the expression of Id-1 alone was upregulated during human keratinocyte differentiation while Id-2 and Id-3 expression patterns remained unchanged. Immunoblotting for Id-1 confirmed the upregulation of Id-1 expression. Id-4, a neuronal-specific Id protein, was not expressed in these cells. Given these results, Id proteins were overexpressed in primary human keratinocytes to determine their ability to alter proliferation and differentiation.

Id genes were introduced into primary human keratinocytes by either plasmid transfection or retroviral infection and cells were grown in selection media to obtain pure preparations of Id-1 expressing cells and subsequently as pools of selected colonies. Cellular lifespan was recorded for pools of transfected cells (Table 1).

TABLE 1
Id Overexpression Extends the
Lifespan of HFKs

Transfectant	Lifespan	Average Lifespan
Neo	1 mo, 2 mos, 1 mo, 2 mos	1.5 mos
Id-1	1 yr, 8 mos, 6 mos, 6 mos	Immortalized
Id-2	3 mos, 3 mos, 4 mos, 4 mos	3.5 mos
Id-3	2 mos, 3 mos, 4 mos, 4 mos	3,25 mos

These data were repeated in six separate experiments and demonstrated a clear extension of keratinocyte lifespan in Id overexpressing pools of cells versus vector controls. In particular, it was noted that Id-1 overexpressing cells possessed an unlimited lifespan in cell culture and were therefore determined to be immortalized. These experiments were repeated using an Id-1 retroviral expression construct and similar results were obtained.

Id overexpressing cells were induced to differentiate under a serum/high-calcium stimulus four weeks following transfection, and differentiation was determined by marker protein expression. While Id overexpressing cells did express small amounts of differentiation marker proteins without a differentiating stimulus, the level of expression did not increase under a differentiation stimulus as seen in control cells. Interestingly, Id-overexpressing cells grown under differentiating stimuli showed many focal regions of poorly differentiated cells within regions of more differentiated cells which was not seen in control transfected cells.

Thus, Id-1 was able to immortalize primary human keratinocytes.

The disruption of p53 and pRb growth regulatory pathways in these cells was then tested. DNA damage was induced by growing Id-1 immortalized keratinocytes in the presence of actinomycin D, and p53 expression was determined by immunoblotting. These data demonstrated abrogation of p53 upregulation in

response to DNA damage in Id-1 immortalized cells. Fluorescence activated cell sorting (FACS) analysis of these cells revealed a G1 growth arrest in response to DNA damage in normal human keratinocytes which was absent in Id-1 immortalized cells. This effect appears to be a late response to Id-1 overexpression in primary human keratinocytes, as keratinocytes overexpressing Id-1 have a normal p53 response to DNA damage early after transfection. We also tested the G1 cell cycle arrest in response to TGFß in these cells as a measure of the integrity of the retinoblastoma growth regulatory pathway. These data demonstrate a G1 growth arrest in Id-1 overexpressing cells similar to that in normal HFKs.

From the above experiments we have determined that Id-1 expression is upregulated during human keratinocyte differentiation, and is believed to function as a cellular immortalizing oncogene when overexpressed in primary human keratinocytes. While Id proteins are typically thought of as being inhibitory to differentiation, the upregulation of Id-1 during this process can represent a titration phenomenon with respect to E-protein expression in these cells during differentiation. This conclusion is supported by recent work which demonstrates E-protein overexpression can lead to induction of expression of the cell cycle inhibitory protein, p21 (Prabhu, S. *et al.*, *Mol. Cell Biol. 17*:5888-5896 (1997)). Furthermore, such p21 induction can be abrogated by overexpression of Id-1. Given that p21 has been demonstrated to be upregulated during keratinocyte differentiation, (Missero, C. *et al.*, *Proc. Natl. Acad. Sci. USA 92*:5451-5455 (1995)), it is likely that Id-1 upregulation is responsive to E-protein expression. Most interesting, is the determination that Id-1 alone is able to immortalize these primary human cells. The mechanism by which Id overexpression can lead to abrogation of p53 DNA damage responsiveness remains to be determined.

While several viral oncoproteins have been demonstrated to immortalize primary human cells as single oncogenes, this is the first demonstration of a cellular oncogene functioning alone to induce primary human cell immortalization. These data suggest that aberrant expression of Id proteins can be a potent oncogenic stimulus in other primary human cells and that Id-associated mechanisms of cellular immortalization can represent a novel pathway for circumventing normal cellular growth controls.

Thus, the invention relates to methods of altering Id expression in keratinocytes. In one embodiment, the invention relates to a method of immortalizing a primary mammalian cell comprising overexpressing an Id protein in said cell. The cells which can be subjected to this method are primary (i.e., non-immortalized) cells, such as normal diploid or somatic cells. Examples of mammalian cells of the invention include epithelial cells, connective tissue cells, muscle cells and nerve cells. In a preferred embodiment, the cells can be keratinocytes, epidermal cells, red blood cells, white blood cells, smooth muscle cells, neurons, and fibroblasts. The cells are, typically, mammalian, including human, rodent (such as murine or rat), primate (such as monkey, chimpanzee or ape), feline, porcine, ovine, bovine, etc. Preferably, the cells are human. Thus, the method includes the steps of maintaining the cells under appropriate conditions for growth and culture, as is generally known for mammalian cells or the specific cell-type, where the invention is conducted *in vivo*.

In a preferred embodiment, the Id protein of the method can be, for example, Id-1, Id-2, and/or Id-3. More preferably, the Id protein is Id-1. Typically, the Id protein is derived from the same species as the cell. For example, the protein can be native to the cell (i.e. possessing the same sequence as the Id protein produced by the cell and/or produced by DNA obtained from the subject who donated the cell) or the protein can be an allelic variant of the protein native to the cell. The protein, or DNA encoding the protein, can also be obtained from a different species or subject or can be synthetic. In yet another embodiment, the Id protein or DNA is not naturally occurring but is a functional mutant or active fragment of the native protein. "Active fragment" or "functional mutant" is defined herein to include Id proteins which, upon overexpression in the cell, result in the immortalization of the cell. Active fragments generally include proteins which possess significant regions of the native protein (e.g. over at least about 50%, preferably at least about 75% or at least about 90% of the total sequence). Mutants generally include proteins which possess significant homology or sequence identity with the native protein (e.g., over at least about 75%, preferably at least about 90% or at least about 95%). For example, the sequence of the mutant is substantially or essentially the same as the native protein. Mutants can be characterized by one or more deletions, substitutions

and/or insertions in the amino acid sequence. Mutants also include fusion proteins containing a sequence which possesses Id protein activity. For example, the Id protein can be fused to a marker to facilitate the determination of the level of protein expression, e.g. visually or immunogenically.

The isolation and mutation of polynucleic acid molecules from cDNA and genomic DNA libraries is well known in the art and is described, for example, in Sambrook, Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989), the contents of which are incorporated herein by reference in its entirety. See Chapters 9 and 15 therein, for example.

Functional mutants and active fragments can be identified by contacting the mutant or fragment with the cell (e.g., by expressing the mutant or fragment in the cell) and determining the level of expression and/or the cell immortalization.

The invention includes altering the level of expression of the Id protein selectively or non-selectively, directly or indirectly. Selective alteration of protein expression is defined herein to refer to the direct manipulation of the Id protein expression alone. That is, a compound which "selectively alters" expression of Id-1 does not alter the direct expression of other proteins, such as Id-2. It is understood, however, that the selective alteration of expression of Id-1 can, in turn, result in the alteration of other proteins in the cell. Selective overexpression or enhanced or increased expression can be achieved, for example, by transfecting with or introducing into the cell a vector which consists essentially of a DNA which encodes the Id protein under operational control of a promoter. It is understood that a vector which additionally contains selectable markers and other sequences which, for example, facilitate replication or chromosomal integration are also embraced within this definition.

Thus, enhancement or activation of Id-1 expression can include the steps of introducing one or more DNA molecules into the cell which result in increased levels of Id protein expression, such as sequences which encode the Id-1 protein under operational control of a promoter. The DNA can additional include enhancer sequences, multiple copies of the gene, selectable markers, sequences which promote chromosomal integration or homologous recombination. The promoter sequences can include any sequence which is recognized by the cell and activate

transcription. Examples include the native or natural promoter region associated with the Id protein. Methods for the expression of cloned genes in mammalian cells is also generally known in the art and are described, for example, in Sambrook, *supra*, Chapter 16.

Immortalization is "activated" or "enhanced" in a cell or population of cells wherein the number of cells which undergo immortalization is increased (e.g., the percentage of cells which undergo immortalization) and/or the time in which the cell or cells require to undergo immortalization is decreased.

Alternatively, the expression of Id protein can be inhibited or reduced by recombinant DNA technology. For example, the gene(s) expressing the Id protein(s) can be deleted or "knocked out". In another embodiment, the sequence can be inactivated, such as by modifying by, for example, a point mutation, which interferes with the expression of functional protein (for example, inserting or deleting an amino acid to shift the open reading frame of the coding sequence). Alternatively, regulatory regions of the coding sequence can be removed or replaced to control expression levels. In yet another embodiment, sequences (or genes which express proteins) which activate Id protein expression can be removed or inactivated. The inactivation, inhibition or reduction of Id protein expression can inhibit the ability of the cell or cells to undergo immortalization. Immortalization is "reduced" in a cell or population of cells wherein the number of cells which undergo immortalization is reduced (e.g., the percentage of cells which undergo immortalization) and/or the time in which the cell or cells require to undergo immortalization is increased.

Methods for transfecting or introducing DNA into mammalian cells are generally known in the art and are described, for example, in Sambrook, *supra*. Additionally, several of the references described above disclose the transfection and/or introduction of DNA into cells. The contents of each of these references are incorporated herein by reference in their entirety.

In yet another embodiment, the Id protein expression is altered by contacting the cell with a compound which activates, enhances or inhibits the expression and/or protein activity. For example, the compound can be a small organic molecule, nucleic acid or a protein. Examples of nucleic acid molecules which can alter

protein expression can include antisense polynucleic acid molecules. Examples of proteins which can inhibit or reduce protein activity include anti-Id protein antibodies. Proteins which can increase Id protein activity can be anti-idiotypic antibodies against anti-Id protein antibodies. Other molecules or compounds which result in the ability to manipulate the immortalization of a cell can be identified by screening, such as a high through-put screen employing, for example, a chemical library or collection of compounds, as is generally known in the art.

Thus, the invention further relates to a method of determining the activity of a compound to alter Id protein expression in a cell comprising contacting said cell with said compound and observing the level of expression of said Id protein. The cells and proteins are as described above. The assay can be in the presence or absence of one or more controls. The invention further relates to compounds obtained from the assays described herein and the use of these compounds in methods of altering Id protein expression *in vitro* and in therapy.

The level of expression can be observed, for example, directly, indirectly or visually, such as in the observation of the cell's ability to immortalize. Alternatively, the level of protein expression can be determined via hybridization of the mRNA (or the corresponding cDNA) to a probe corresponding to a coding sequence (or a fragment thereof which selectively hybridizes) of the DNA encoding the Id protein, or the complements thereof. In yet another embodiment, the level of protein expression can be determined immunogenically by contacting a cellular protein fraction with an antibody which binds Id protein or to an immunogenic determinant fused to the Id protein or co-expressed with the Id protein, as can be manufactured via recombinant DNA technology. Alternatively, one can employ promoter-reporter gene fusions, (such as, chloramphenicol acetyl transferase, luciferase or β-galactosidase). Other methods of determining protein level will be immediately apparent to those skilled in the art.

It can be desirable to immortalize cells to facilitate maintaining the cells in culture. That is, immortalized cells generally reproduce and grow faster and for longer periods of time than normal diploid cells. This can be advantageous in storing and/or culturing the cells and its progeny for extended periods of time. The conditions for maintaining the cells in culture can be those generally employed in

the art in relation to mammalian cells or the specific cell type. Furthermore, immortalized cells obtained by this method can be used generally in assays for cell-based systems, thereby avoiding difficulties associated with manipulating normal diploid cells. The invention has advantages over known systems for immobilizing cells in that it does not require the use of viral sequences (e.g., a retrovirus or DNA tumorvirus) which immortalize cells. It is noted, however, that DNA encoding an Id protein can be delivered to the cell by a viral vector. Additionally, the cells produced by the methods described above can be used as a source of tissue or cell specific mRNA or cDNA or genomic DNA, as is known in the art. In yet another embodiment, the cells can be used as host cells for the production of recombinant and non-recombinant proteins.

Immortalization of cells *in vivo* or the transplantation of immortalized cells can be desirable in the development of animal models for tumor growth. That is, the method can be utilized to induce tumor growth in a selected cell or cells in an animal (such as in a mouse, rat or primate). Such animal models are useful in assays for anti-tumor agents or therapies, employing well known techniques. As such, the invention further relates to these animal models, methods of using them in screens for therapeutic agents and the therapeutic agents identified by these screens. Additionally, the invention can be employed in screens for compounds that induce tumors (accelerate the genesis of tumors in systems), e.g., in a toxicology screen.

Likewise, the above method can be used to inhibit or reduce immortalization of cells *in vivo* or in culture, where it is desirable to maintain and manipulate normal diploid cells. Thus, in cells which can or are expected to undergo immortalization where it is undesirable (for example, in assays studying the cellular functions of an immortalized cell, or where the cell or cells are expected to be employed in therapy (e.g. a tissue transplant or graft)), immortalization of the cells can be inhibited by this method. Additionally, compounds which inhibit Id overexpression can be useful in preventing the formation of tumors in animals at risk thereof.

In yet another embodiment, the method of the invention can be used to study the differentiation and cell growth of mammalian cells. As such, the interactions of cellular products, including the bHLH DNA binding proteins, E-proteins, growth factors and other proteins implicated in cell cycle transitions can be studied and

further elucidated employing the methods of the present invention. These methods can be used to determine the functions of these proteins in cell cycling and in diseases implicated in cell cycling (e.g. cancer), thereby obtaining therapeutic targets and therapeutic agents, which are also envisioned as being an embodiment of the claimed invention.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

CLAIMS

What is claimed is:

1. A method of immortalizing a primary mammalian cell comprising overexpressing an Id protein in said cell.

2. The method of Claim 1 wherein said primary mammalian cell is a keratinocyte.

3. The method of Claim 2 wherein said Id protein is Id-1.

4. The method of Claim 3 comprising the steps of transfecting said cell with a DNA molecule which codes for said Id-1 protein under operational control of a promoter.

5. The method of Claim 3 comprising the steps of contacting the cell with a compound which activates or enhances Id-1 protein expression.

6. The method of Claim 3 wherein said overexpression of said Id-1 protein is selective.

7. A method of inhibiting the immortalization of a primary mammalian cell comprising inhibiting expression of an Id protein in said cell.

8. The method of Claim 7 wherein said primary mammalian cell is a keratinocyte.

9. The method of Claim 8 wherein said Id protein is Id-1.

10. The method of Claim 8 comprising the steps of transfecting said cell with a DNA molecule which inactivates the DNA which codes for said Id-1 protein.

11. The method of Claim 8 comprising the steps of contacting the cell with a compound which inhibits Id-1 protein expression.

12. A method of determining the activity of a compound to alter Id protein expression in a cell comprising contacting said cell with said compound and observing the level of expression of said Id protein.

13. The method of Claim 12 wherein said primary mammalian cell is a keratinocyte.

14. The method of Claim 13 wherein said Id protein is Id-1.

15. The method of Claim 14 further comprising the steps of transfecting said cell with a DNA molecule which codes for said Id-1 protein under operational control of a promoter.

16. The method of Claim 14 wherein said Id protein is activated or enhanced.

17. The method of Claim 14 wherein said Id protein expression is inhibited.

INTERNATIONAL SEARCH REPORT

Int'l Application No
PCT/US 99/00020

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 693 487 A (BLACKWOOD E.M. ET AL.) 2 December 1997 see the whole document ---	1-17
A	JENKINS T D ET AL: "The keratinocyte -specific Epstein-Barr virus ED-L2 promoter is regulated by phorbol 12-myristate 13-acetate through two cis-regulatory elements containing E-box and Kruppel-like factor motifs." JOURNAL OF BIOLOGICAL CHEMISTRY, (1997 SEP 26) 272 (39) 24433-42, XP002102954 see the whole document --- -/--	1-17

[X] Further documents are listed in the continuation of box C.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	VASTRIK I ET AL: "Expression of the mad gene during cell differentiation in vivo and its inhibition of cell growth in vitro." JOURNAL OF CELL BIOLOGY, (1995 MAR) 128 (6) 1197-208, XP002102955 see the whole document	1-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte :onal Application No
PCT/US 99/00020

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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